Maximizing the applicability of continuous wave (CW) Electron Paramagnetic Resonance (EPR): what more can we do after a century?

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Abstract

Electron Paramagnetic Resonance/Electron Spin Resonance (EPR/ESR) spectroscopy is a powerful experimental approach solving challenging problems that are inaccessible to other experimental techniques. While pulsed EPR techniques and their applications have been the central focus of the field nowadays, methodology development and application of continuous wave (CW) EPR have never been undervalued. Although the importance of EPR in research has been recognized in many areas as indicated by the increasing number of EPR research articles published every year, the growth in the number of EPR research teams seems to not match the former, likely caused by the relatively high cost of modern pulsed EPR spectrometers. In this perspective, we aim to discuss the new areas and new directions of the application of CW EPR in combination with spin labeling that are slightly different from the classic/traditional applications of the CW EPR technique. Based on our experience, we show example applications of CW EPR on the interfaces of protein-confinement materials, protein-inorganic crystal lattices, protein-nanoparticles, and protein-polymers. Various structure and dynamics information can be resolved from the data analysis of CW EPR, leading to enhanced understanding of protein biophysics upon interaction with the synthetic nanostructures as well as possible guidance of hybrid materials development based on the combination of proteins and nanostructures. We also discussed the possibilities of even broadening the application spectrum of EPR spectroscopy. The relatively high affordability of CW EPR spectrometers (as compared to the pulsed ones) also facilitates the growth in EPR research groups worldwide.

1. Introduction

Electron Paramagnetic Resonance (EPR)/Electron Spin Resonance (ESR) is a powerful tool sensitive to unpaired electrons. Starting with detecting, quantifying, and understanding paramagnetic species in natural and synthetic materials, more interesting and sophisticated systems/problems have been found to be accessible only to EPR. All these achievements rely on the development of various EPR-based methodologies, including continuous wave (CW) EPR (mostly in the early days but also widely applicable as of now) as well as pulsed EPR, which has played an important role in modern EPR research. Both EPR approaches have led to the solution of a large number of challenging problems dealing with natural/artificial paramagnetic species and, more importantly, complex biological systems every year. However, the rate of increase in EPR research articles does not match (and often, is far below) the rate in the increase in the number of EPR research groups, in spite of the decent number of EPR Ph.D.s graduated world-wide each year. One possible cause may lie in the relatively high cost of modern EPR spectrometers, especially the widely applied/needed, pulsed EPR spectrometers. Such a cost becomes a practical barrier limiting the research institutions who intend to promote/invest on EPR research (and hire EPR faculties), especially among the large number of medium-small sized institutions, regardless how clear the needs of EPR in various fields research have been demonstrated. Consequentially, such a reality may be one reason that makes establishing an EPR-focused independent research group more competitive and challenging, which may also discourage the next generation of EPR scientists to start an independent career.

While one solution is to compete for the limited openings in large-sized institutions, which is another practical barrier, it is possible to home-build pulsed EPR spectrometers with a lower budget. However, because most labs already have EPR spectrometers built/purchased and directly

applicable to research, students usually have limited experience in home-building a pulsed EPR spectrometer during the Ph.D. or postdoctoral training. Home-building an instrument also costs the precious time of tenure clock for young principle investigators (PIs). Another alternative is perhaps to start with a more affordable equipment for most research institutions, a CW EPR spectrometer, and maximize the applicability of it. The challenge, of course, lies in convincing the institutions that even a CW EPR spectrometer is powerful enough to lead to, at least, the start of an independent research group and solve important problems. Another challenge of this strategy is that CW EPR has been developed and well-known for so long that new directions and new areas are not so straightforward to define or explore.

In this perspective, we aim at summarizing the technical development of CW EPR methods, key areas of the current applications of CW EPR, and most importantly, by show-case, the new research areas that CW EPR can be applied to based on our recent experience. Our purposes are to introduce the use of the technique in more areas and offer new perspectives to conduct EPR research as an academic career (to eventually help promote the growth in the number of EPR research groups in small-medium sized institutions world-wide by avoiding asking for a high startup budget). Due to the large (in fact, huge) body of literature in EPR, with sincere apologies, we can only cite a limited number of references to avoid over-citation. However, all EPR seniors' and pioneers' work is greatly appreciated during our research and through this perspective.

2. CW EPR is a powerful tool.

The methodology and application of CW EPR spectroscopy are developed orthogonally supporting each other, forming a 2-dimentional diagram (*i.e.*, the z-axis and y-axis of Figure 1). Starting with the z-axis, typical information obtained from a CW EPR experiment includes the nature of a paramagnetic species center (metals or radicals), the quantity of the present

paramagnetic species, and how environment influences the species.[1-3] In addition to the sensitivity to unpaired electrons, EPR also offers a unique advantage, isolating and probing only the unpaired electrons regardless of the surrounding nuclei or sample phase (the lossy effect of samples in aqueous environment at room temperature, which is due to dielectric losses, and thus the properties of the electric dipoles of the water molecules, has to be minimized by reducing the sample volume).[4, 5] The obtained spectra can usually be analyzed using simulation algorithms based on first-principal theories, the most widely used of which is based on the microscopic ordering and macroscopic disorder (MOMD) model developed by Freed and coworkers, which unambiguously reveals the correlation between CW EPR spectrum and the rate/order of the motion of the spin probe/label.[6, 7] Several user-friendly software packages such as EasySpin and Multicomponent are also available to facilitate and promote the application of CW EPR data analysis, leading to important structural (polarity, for example) and dynamics (at the sub-nanosecond scale) information.[8-10] CW EPR can often be carried out in a time-resolved mode, facilitating the monitoring of live processes in real time. In combination with power saturation, CW EPR is able to probe the spin-lattice relaxation time and relevant dynamics information (often slower than ns).[11-15] CW EPR is also sensitive to the spin exchange interactions when more than one electron spins are present in a close proximity (usually a few Å), reporting the interactions among spins.[13, 16-18] EPR oximetry on nitroxide spin probes/labels relies on the detection of the collision of nitroxide and molecular oxygen, which reflects the local polarity.[19-22] This is another method to probe heterogeneous environments (*i.e.*, lipid bilayer membranes) and found clinical applications.[23, 24] For two spins separating by 5-25 Å, the magnetic dipolar interaction between the spin centers may be extracted (a software available, ShortDistances, by Dr. Altenbach),[25] leading to the distance distribution between the centers.[26] More recently, high

hydrostatic pressure technique has been developed EPR studies, leading to the sense of "invisible" protein conformations and their exchange with the native protein states.[27-29]

The methodology development listed above has often been combined with (site-directed) spin labeling (SDSL) of the target (bio)macromolecules, leading to a wide spectrum of application in biological and synthetic materials problems (y-axis of Figure 1). SDSL of proteins significantly broadens the application of EPR, wherein CW EPR offers the local dynamics of the labeled protein and thus, reports the local polarity, crowding, and changes in these upon alteration in the environment.[30-38] A key advantage is, again, to isolate the labeled site of protein for study without interference of the surrounding biomolecules. This advantage allows the study of transmembrane proteins wherein the presence of lipids and the heterogeneity in sample phase challenge most other experimental techniques for protein studies. Herein typical information obtained from CW EPR includes the depth of a residue being buried inside of the membrane bilayers and the helical structure of the transmembrane domain based on the water and/or oxygen accessibility probed by CW EPR.[31, 32, 39-41] Nucleic acids can also be spin-labeled and CW EPR offers the similar information, local polarity and crowding as well as changes in these upon interaction with DNA-binding proteins or other nucleic acids.[42-44] Other complex systems such as the protein-protein, protein-DNA complexes, and membrane proteins are also studied by CW EPR and SDSL.[34, 43, 45-47] Polymers, although relatively rare, can also be spin labeled site-specifically at the desired position, leading to local dynamics, crowding, and polarity, reporting important information that explains the properties of polymers.[48-56] Of course, CW EPR can be used to probe the metal centers in metalloproteins without artificial spin labeling. Lastly, CW EPR has been applied in materials studies to reveal materials formation mechanisms and/or structure-property relationships.[57-59] Thus, as shown in the 2-dimensional (2D) diagram (Figure 1 y-z

plane), CW EPR methodology development offers a fruitful application in various fields, thanks to all the excellent works by the EPR pioneers.

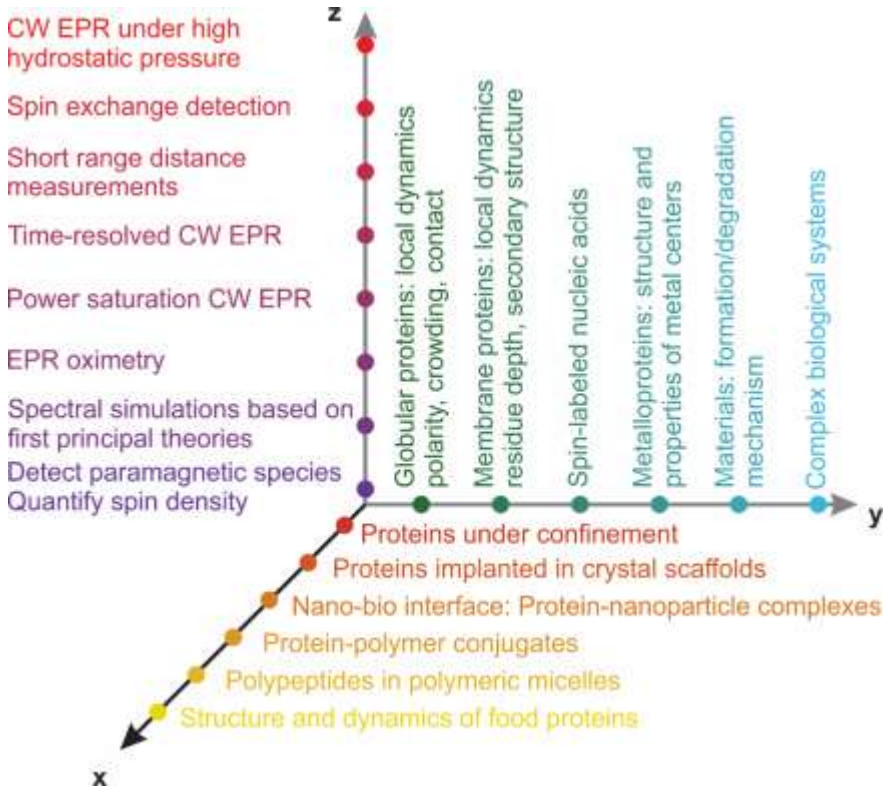

Figure 1. Illustration of the current CW EPR methodology (z-axis), typical applications (y-axis), and new areas being explored (x-axis) that are discussed in this work.

3. New voices in magnetic resonance: new directions to maximize the applicability of CW EPR.

Our team is constantly looking for new applications of CW EPR in combination with (bio)macromolecular spin labeling since the beginning of the PI's independent career. Because new proteins and new complex biological systems are constantly reported, directly applying CW EPR to these systems, which would continue elongating the y-axis of Figure 1, will with no doubt

provide unique information to offer in-depth understanding of these new biological systems. However, this strategy may be limited by the difficulty in synthesizing or accessing these new biological systems in a reasonable time period for a young group. Thus, while never stop being interested in complex biological systems and publishing via collaboration with biochemistry teams,[60-64] we have also attempted to explore another dimension, the interface of biomacromolecules and nanoscale synthetic materials (nanostructures), or, the interface of two "kingdoms" of the modern world. The original motivation was to develop and understand hybrid materials that simultaneously possess the advantages/ properties of both biological molecules and synthetic materials, namely the biological activity/specificity and the thermal/mechanical stability, respectively. Later on, during our research, we found it possible to endow nanostructures new roles so that they can mimic the natural counter part of protein interactions, such as the cellular protein crowders (nanoparticles) and certain cellular compartment (porous materials), if the synthetic nanostructures are designed and selected properly. The obtained knowledge will allow for cell-free investigation of many important cellular processes without the interferences of the complex cellular components and interactions, shedding light on deepening the understanding of relevant biological processes and the development of therapeutic approaches against the malfunction of these biological processes. Such a combination of hybrid materials and molecular biophysics research not only leads to a number of interesting discoveries but also enhances our team's productivity. Below we summarize some key findings in our exploration of the third dimension of Figure 1.

3.1 Proteins under confinement in porous materials.

Confinement restricts the protein within a limited 3-dimensional (3D) space, a different environment as compared to the dilute solutions wherein most protein studies were carried out,[65-

67] but closer to the cellular conditions where proteins perform their functions. The cellular confinement possesses a high heterogeneity depending on the protein of target and the species/organisms of the cell.[68] A cellular organelle/compartment can be as large as several hundreds of nm, yet due to the high density of cellular proteins, the actual confinement a protein "feels" in a cell is usually similar in size to the protein itself, or, on the order of nanoscale due to the surrounding cellular proteins.[69] Also, molecular chaperones and channel-like proteins place nm scale confinement on proteins hosted therein. Thus, it is meaningful to understand how nanoscale confinement impacts the interplay of protein structure, dynamics, and function. However, it is challenging to investigate the high heterogeneity of cellular confinement on proteins immediately due to the complex cellular components and their interactions;[70] mimicking the nanoscale confinement using synthetic materials becomes a promising alternative. Although proteins immobilized in mesoporous materials have been investigated in the literature, most current studies were focused on the stability and function of the immobilized proteins;[68, 71-74] the structural basis of the function alteration has been underexplored. Recent advances in synthetic porous materials offer an opportunity to probe the influence of each confinement factor, such as the size, shape, and hydrophobicity/charge of the pores as well as their interplay on proteins. Such a fundamental study could possibly lead to a comprehensive understanding of how the complex cellular confinement impacts proteins.

Keeping this in mind, we have explored a few example porous materials for protein confinement. We initiated our investigation by tuning protein encapsulation in mesoporous silica-nanoparticles (mesoSiNPs).[75] The commercial mesoSiNPs were loaded with a spin-labeled model protein, lysozyme (lys), followed by removed of surface adsorption proteins under high ionic strength. CW EPR was then applied to probe the contact of each labeled residue with the

inner channels of the mesoSiNPs, which usually results in a broadened spectrum as characterized by high order and slow motion parameters upon spectral simulation.[75-77] The orientation of the protein inside of the channel can be heterogeneous, meaning the same labeled site may also not contact the channel, which usually results in a second spectral component as characterized by a faster motion and less restriction in the motion (see Figure 2a "m" and "im" components, respectively). Via a thorough spectral simulation, we found the relative contribution of the two cases for each labeled site (6 surface sites total), which led to the proposed protein orientation inside of the mesoSiNP channels (Figure 2b). Interestingly, by tuning the surface charge of the channels, it is possible to alter the interaction between lys and the channel, and thus, tune the encapsulation of the protein. Here, CW EPR serves as a powerful way to probe the orientation of a protein inside of a confined environment.

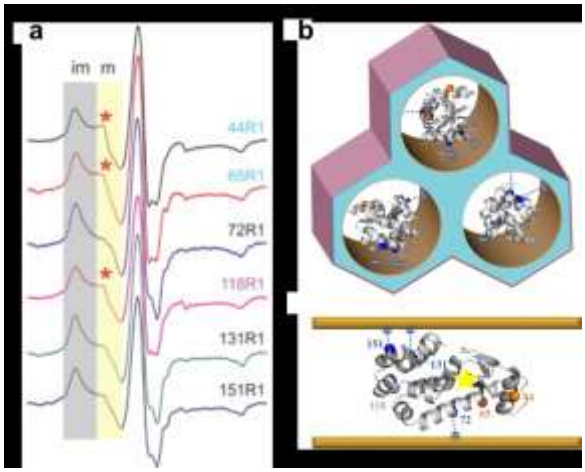

Figure 2. (a) CW EPR data on six surface residues spin labeled with a nitroxide. * indicates spectral components that is originated from a label not in contact with the channel. (b) Illustration of the protein relative orientation in the channels of the mesoporous Silica-nanoparticles. Dotted lines indicate relatively stronger interaction between the labeled residue and the channel. Star = lys active site. Figure obtained from reference with permission.[75]

One of our recent works continued this research direction but employed a new series of channel materials, the Covalent-Organic Frameworks (COFs), which offer more uniform and tunable channel properties.[78] Here COFs together with Metal-Organic Frameworks (MOFs) are considered to be advantageous mesoporous materials to host protein enzymes.[79-86] Our goal was to probe the subtle differences in channel hydrophobicity/ hydrophilicity on protein structure-function relationship. In doing so, lys was loaded into a series of COFs with increasing hydrophilicity, namely COF-OMe, COF-OH, and COF-ONa, wherein the channel diameter is 3.4 nm for all COFs but the surface was modified with the -OMe, -OH, and ONa functional groups, respectively.[78, 87] The diameter is close to the size of lys (2x3x4.5 nm) and was expected to influence lys structure and function. The catalytic activity of lys against a small substrate that can diffuse into the channels[88] decreased as the channel became more hydrophilic, a surprise for the hydrophilic protein lys (Figure 3 inset, left). We, therefore, employed CW EPR (representative data see Figure 3 inset, right) to probe the possible structural basis of such a surprise and found that, as the channel became more hydrophilic, more labeled sites show spin exchange interactions in the EPR spectra (Figure 3 a&b).[78] We rationalized the spin exchange to the strong interaction of the protein with the conjugated COF channels so that the unpaired electron can hop onto the conjugated COF backbones (due to the aromatic functional groups of the COF building blocks); for more than one protein molecules in the same channel, the unpaired electrons can “travel” along the COF channel walls and be “exchanged” with each other. Thus, such an enhanced number of sites that strongly contact the hydrophilic channels can lead to an enhanced restriction to the conformational degree of freedom (Figure 3c), resulting in the reduced catalytic efficiency

observed in our activity test. Here CW EPR probes the spin exchange interactions and reports the relative strength of how proteins contact the synthetic nanoscale channels.

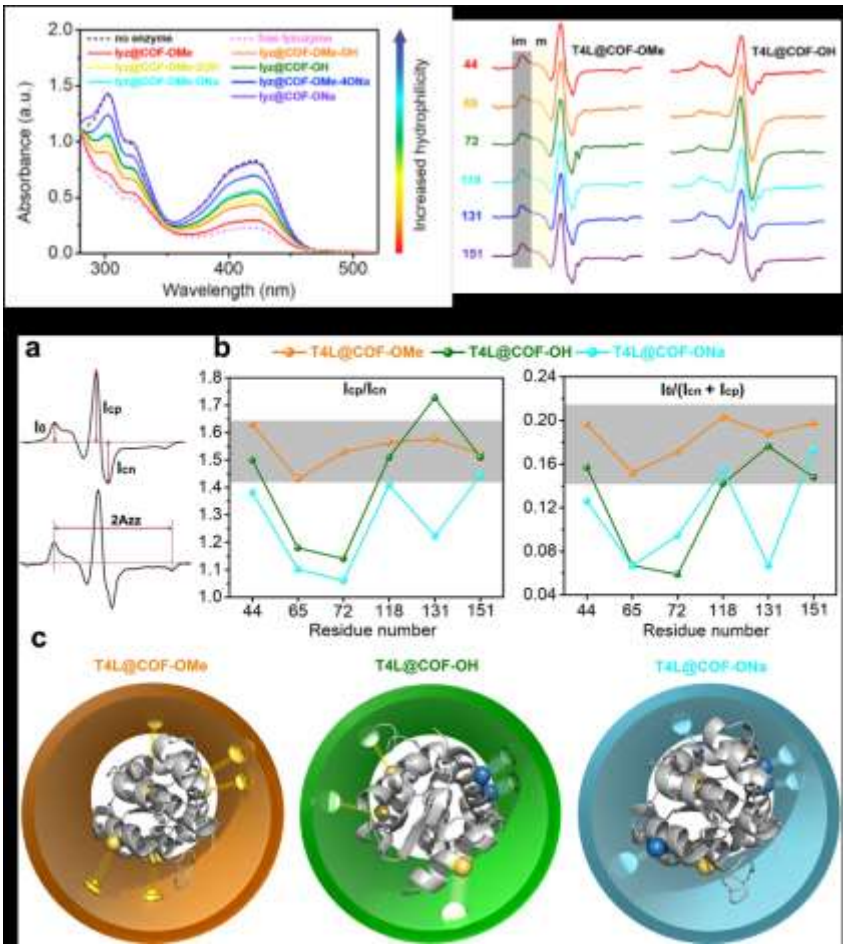

Figure 3. (a) Illustration of quantitative analysis of the spin exchange interactions. (b) Exchange parameters for each labeled lys site defined in (a). (c) Schematic drawings of lys interaction with different COF channels. Dotted lines indicate relatively weak interaction while solid lines indicate strong contact with the channel. (inset) The catalytic efficiency of lys in different COF channels (left) and representative CW EPR data on six surface sits of lys (right). Figure obtained from reference with permission.[78]

We also explored the physical process of protein loading into nanoscale channels in real time, with a special focus on the orientation changes during the loading. Depicting the protein orientation changes in relative to the channel surfaces not only mimics the mechanism of protein transfer into nanoscale compartments in cells but also is of fundamental importance in understanding the potential protein alteration during protein trafficking. The challenge lies in the lack of proper experimental techniques with sufficiently high resolution to depict the protein relative orientation (docking) on the channel in real time, as most time-resolved experimental techniques are challenged by the relatively low resolution which cannot resolve the residues/regions of proteins that contact the channels, and thus, relative protein orientation. In situ monitoring the CW EPR spectral changes in real time on proteins labeled at various surface sites became almost the only approach to offer residue level resolution of protein-channel contact. We, therefore, applied time-resolved CW EPR on spin labeled lys and probed its loading into three channels with distinctive surface properties using mesoSiNP (MCM-41, a negatively charged channel), PCN-128 (a neutral MOF), and COF-ETTA-TPDA (a hydrophobic COF; representative data see Figure 4a). CW EPR data analysis as described in our recent work[76, 77] resolved the relative population of the spectral component (and thus, the chance) of a labeled site to contact a channel at each time point. By probing many time points, we were able to depict the changes in the "contact" component (and the chance of contact) over time for each labeled site (Figure 4b; possible interpretation of data see Figure 4c). This allowed us to depict the orientation of lys (the residues that contact the channel outer surface or inner walls) at each time point, leading to a dynamic picture of how the protein is translocated into each channel in real time. This picture indicated that depending on the surface properties of the channels, different phases/steps of transfer

were observed, with different protein docking model at each phase/step of the transfer.[89] Here time-resolved CW EPR spectroscopy was applied to monitor a biophysical process in real time.

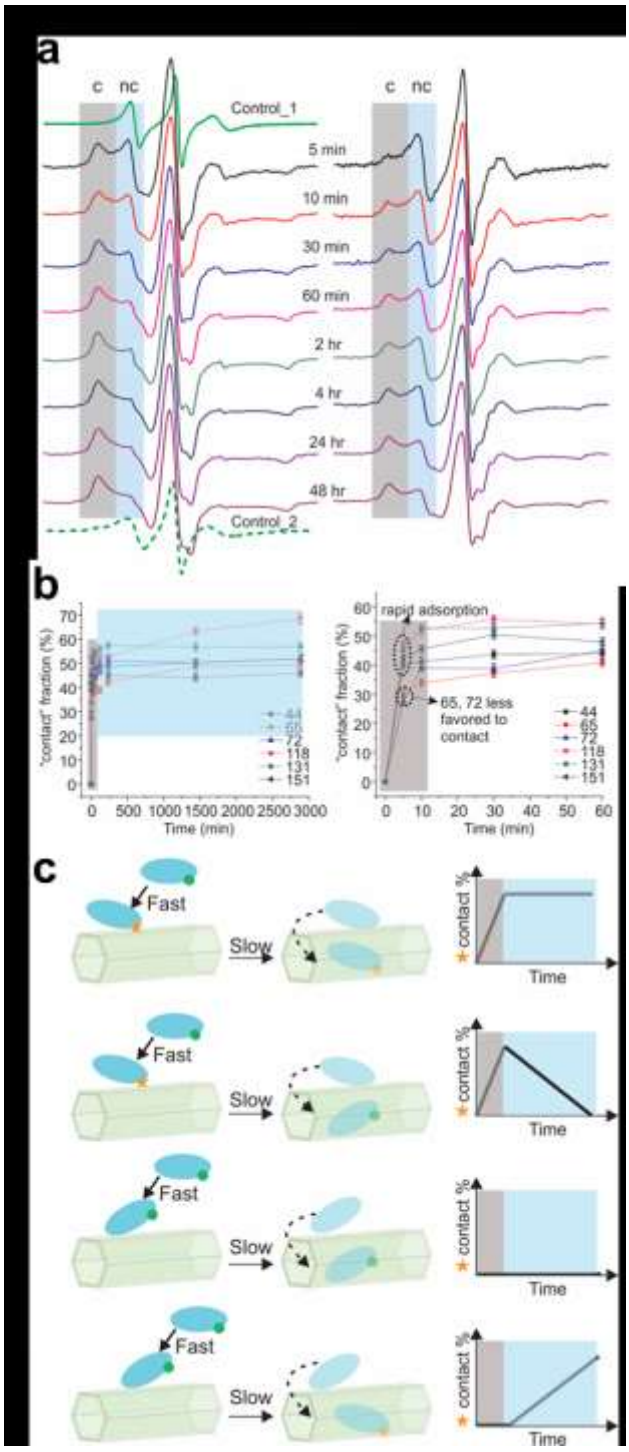

Figure 4. (a) Time-resolved CW EPR data on two representative sites of lys up translocation into a hydrophobic channel, COF-ETTA-TPDA. (b) Spectral simulation on six sites at 8 time points each revealed the relative population of the chance for each site to contact the channel at each time point. (c) Four possible scenarios that can cause the change in the "contact" component over time. The experimental data can be a combination of more than one scenario. Figure obtained from reference with permission.[89]

3.2 Protein "implanted" in crystal scaffolds.

During our research, we found a unique way to immobilize proteins on synthetic materials, the co-crystallization of proteins with certain metals and ligands.[90-92] Often, proteins can serve as the crystallization nuclei to facilitate the formation of metal-ligand framework, resulting in MOFs or metal-organic materials (MOMs), the latter of which differs from MOFs by the lack of 3D scaffold structure (but only 1D or 2D), if defined strictly.[93-96] In this process, proteins are not only encapsulated inside of the crystal scaffolds but also implanted on the crystal surface (for a portion of protein molecules), like a tree with the root under the crystal and the branches/leaves above. Such a unique way of protein/enzyme immobilization provides advantages in biocatalysis especially when the substrate is larger in size than the nanoscale, MOF/MOM pores.[95, 96] A critical factor in this biocatalytic strategy is to ensure that the proper portion of the enzyme is exposed so that the substrate can sufficiently contact the active site of the enzyme. In addition, for the buried enzyme molecules, the backbone dynamics may also influence the biocatalysis if small substrates are applied. CW EPR offers an opportunity to probe both pieces of structural information.

We first noticed such a unique protein immobilization way when preparing enzyme@ Zeolitic-Imidazolate Framework (ZIF-8)[97] by co-crystallization of lys with Zn^{2+} and imidazolate in methanol (MeOH) with the purpose of immobilizing, storage, and controlled release of enzymes (because ZIF-8 can be disassembled in weakly acidic conditions).[90] Upon sonication, high salt, and detergent treatment to remove the lys adsorbed to ZIF-8 surface, we found the lys@ZIF-8 composites were able to degrade bacterial cell walls, the physiological substrates of lys. Because the cell walls are on the order of micrometers (μm), three orders of magnitude higher than ZIF-8 pores (6-7 Å),[97] we realized that some lys must be implanted on the surface of ZIF-8 with (at least partial) exposure of their active site. The same concept was later proved using amylase enzyme and starch, another large substrate that is three orders of magnitude higher than MOF/MOM pores,[91, 95, 96] confirming our proposed "tree"-like enzyme immobilization. We then employed CW EPR and SDSL to probe the chance for each labeled surface residues (typically 6-8 residues were studied to cover most of the lys surfaces). Similar to the mesoSiNP study discussed in 3.1, we found two spectral components originated from a labeled site being 1) exposed to the solvent (faster motion and less spatial restriction) and 2) buried under the crystal surface (slower motion and high restriction).[90] Spectral simulation was then employed to deconvolute the two contribution/population of each, resulting in the chance for a labeled site to be exposed. After scanning a few surface sites, lys regions that are preferred to be exposed can be determined, resulting in the orientation and exposed region of lys on ZIF-8 surface (Figure 5). We found that the N-terminus (represented by residues 44 and 65) likely had a higher chance to be exposed above the crystal surface (Figure 5 orientation I) as compared to the C-terminus; this may explain the observed activity because the active site of the protein is closer to the N-terminus. Also, other orientations (Figure 5 orientation II and III) were possible, although the chance to have these

orientations varied (30-40 % for orientation I and II vs ~10-12 % for orientation III). We later demonstrated the same concept on enzyme@MOF growing on nanotubes,[91] magnetic nanoparticles,[96] and graphene oxides[92] and revealed the exposed protein region in each case using CW EPR.

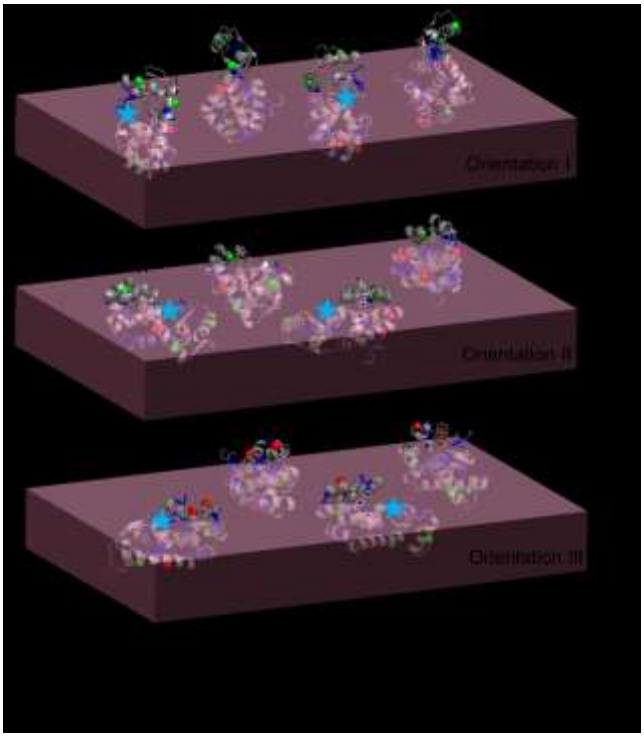

Figure 5. Illustration of protein implanted into the ZIF scaffolds. Three orientations were found possible on the surface of the ZIF crystals with different relative probability. Figure obtained from reference with permission.[90]

Recently, we found it possible to alter the orientation and dynamics of enzymes encapsulated in MOFs/MOMs via co-crystallization by changing metals and ligands, which resulted in altered catalytic performance.[93] For example, we found that Ca-NH_2-BPDC served as an optimal crystal platform to immobilize enzymes as compared to two other Ca-based MOMs,

Ca-BDC and Ca-BPDC, because most tested enzymes displayed a higher catalytic efficiency when encapsulated in Ca-NH_2-BPDC, regardless of the substrate size. To probe the structural basis of this finding, we employed CW EPR to determine the relative dynamics of enzymes inside of each Ca-MOM with a focus on the buried spectral component (the one with slower motion and higher restriction), because most of our tested enzymes have smaller substrates and do not need to be exposed to deliver their catalytic function.[93] CW EPR showed two components for each labeled enzyme site in all three Ca-MOMs (representative data see Figure 6a&b), which was expected since a portion of the enzyme molecules were buried and the rest exposed (Figure 6c). However, we found that the buried component in the Ca-NH_2-BPDC displayed a higher rate of motion and less ordering restriction as compared to the same component in the other two Ca-MOMs (Figure 6d). This was proposed to a possible reason why most enzymes in Ca-NH_2-BPDC displayed a higher activity: the Ca-NH_2-BPDC offered a more friendly environment so that the enzymes can have a higher degree of freedom even though the enzymes were entrapped under the crystal surface, which often facilitates the catalytic function.[93]

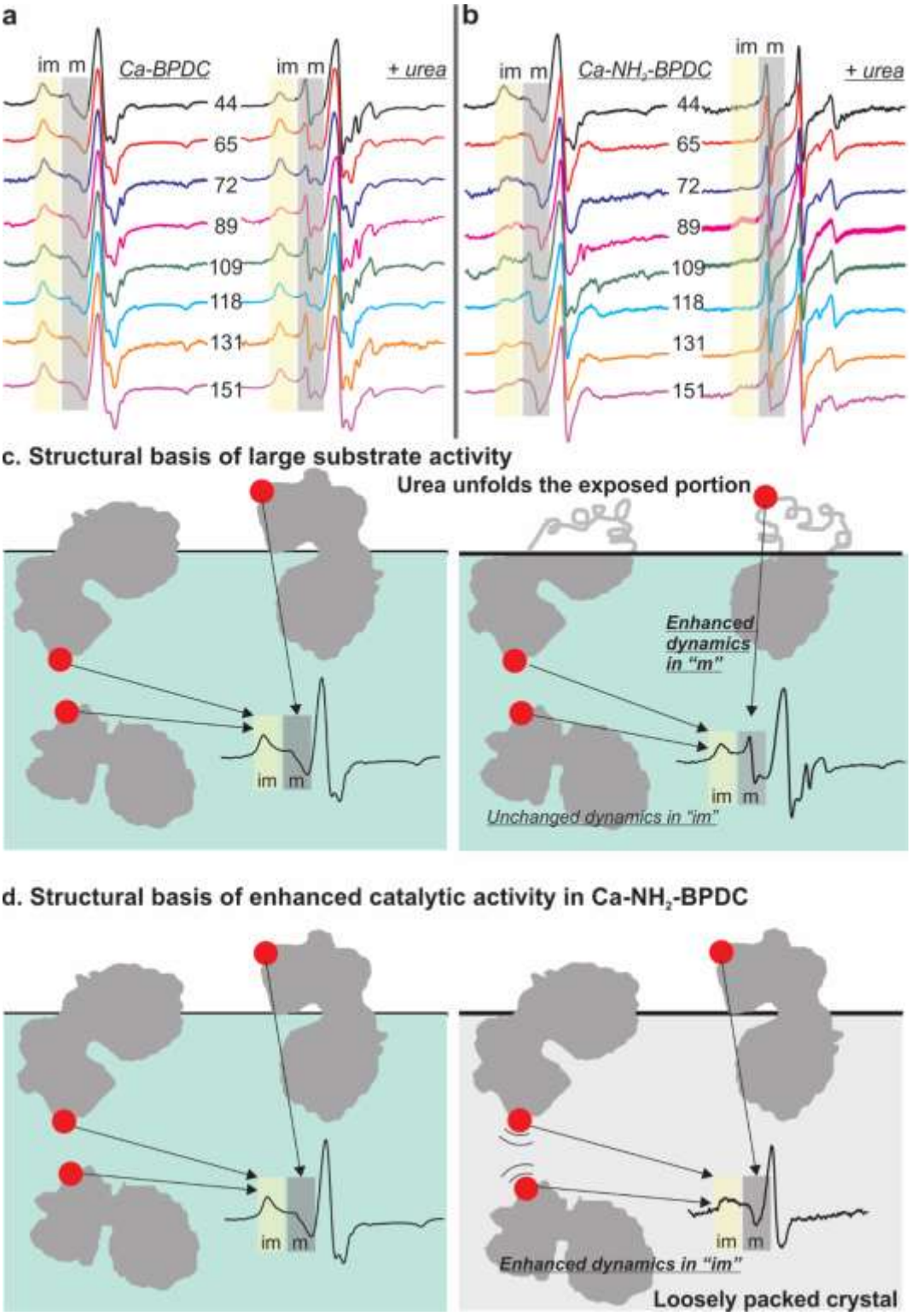

Figure 6. (a,b) CW EPR data on eight surface sites of lys upon implantation into Ca-BPDC and Ca-NH2-BPDC. Urea was added to confirmed the partial exposure of the labeled sites. (c)

Illustration of the confirmation of the partial lys exposure with urea and CW EPR. (d) Illustration of the catalytic efficiency enhancement when the crystal is loosely packed. Figure obtained from reference with permission.[93]

3.3 Protein-nanoparticle interactions: the nano-bio interface.

The original motivation of studying the nano-bio interface is to understand how do nanoparticles influence biomacromolecules, specially proteins, once released into the environment or biological systems, either intentionally or by accident, with a broader impact on public health given the rapid development of nano-technology. Also, from the point view of nanoscience, a general concern is how biological environment and molecules influence the function of nanoparticles and relevant devices once applied in reality. Additional benefit is to offer the structural basis of protein alteration when nanoparticles are designed as protein carriers for therapeutic protein delivery or biocatalysis. In our first attempt, we found that a globular protein, lys, was able to trigger the aggregation of gold nanoparticles (AuNPs) even under an extremely low concentration.[98] This phenomenon was rarely reported in the literature of nano-bio studies, which often show how proteins or other biomolecules dock onto AuNP surfaces for device development or biomedical applications. We realized that our finding may help a general understanding on the impact of globular proteins on AuNPs, and thus, probed the mechanism of such an aggregation. Using CW EPR and SDSL of lys, we found that the protein barely underwent any dynamics or structural changes during the AuNP aggregation, which, in combination with other experimental evidences, suggested that lys serves as the bridge to bringing two AuNPs to close proximity via electrostatic interactions without damaging lys itself. This finding was the first report on such a unique role of globular proteins in causing AuNPs aggregation.[98, 99] CW EPR

offers an opportunity to probe and monitor the dynamics of proteins during the complex aggregation process. On SiNPs, we also applied SDSL and CW EPR which revealed the relative orientation of lys upon docking onto SiNPs with various surface modifications (principles of probing orientation were similar to those discussed in 3.1; Figure 7).[100] Depending on the surface charge (slightly negative on OH-SiNP and more negative on COOH-SiNP; Figure 7) and the initial mixing ratio of protein to SiNP, single layer (Figure 7a&c) or multi-layer (Figure 7b&d) protein corona were formed. For the single layer case, CW EPR revealed the relative orientation of the protein while in the multi-layer case CW EPR was also able to resolve the docking intensity in addition to protein orientation in each layer (tight docking led to slower motion of all labeled sites). Knowing the orientation and the docking mechanism of globular proteins on SiNPs helps understand the nano-bio interface at the molecular level or even higher resolution, shedding light on the rational design of future protein carriers. The strategy of using CW EPR to probe protein orientation and dynamics is applicable to a broader range of nanoparticles.

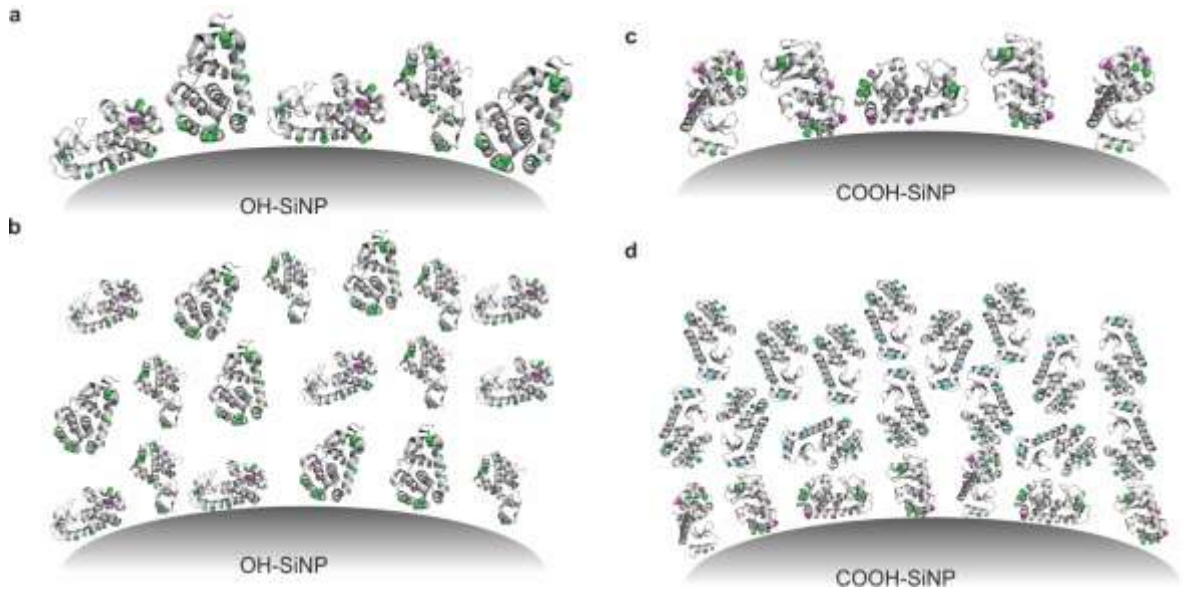

Figure 7. (a,b) Protein orientation on the hydroxyl SiNP surface when a single layer and multi-layer protein corona are formed depending on the protein:SiNP mixing ratio. (c,d) SiNPs with

more negatively charged surfaces display orientation preference and more intensive packing when multi-layer of proteins are coated. Figure obtained from reference with permission.[100]

3.4 Protein-polymer conjugates.

The concept of using polymeric materials to alter protein properties has been proposed and demonstrated in partial.[101] However, the structural basis of how polymers influence the host protein at the molecular or residue resolution was underexplored, likely due to the challenges in probing protein structural and dynamics changes at a sufficiently high resolution using most experimental techniques, given the interference of polymers to the data analysis of these techniques. SDSL and CW EPR are immune of the presence of polymers when probing protein dynamics (as long as the polymer is not paramagnetic), offering an opportunity to fulfill the knowledge gap here. For proof-of-principle, we chemically conjugated polyethylene glycol (PEG) with various lengths and abundance to our model protein, lys, on protein carboxylate residues.[102] We found a correlation between the overall molecular weight of the protein-polymer conjugates (adjusted by changing the PEG molecular weight and abundance) and the rotational correlation time from CW EPR spectral simulation. For most labeled sites, such as correlation validates regardless of the labeling position, which is reasonable since mutation and spin labeling do not change the molecular weight of the conjugates significantly. For two labeled sites, however, we found the correlation was lack, so that increasing the amount of PEG or weight of PEG did not alter the CW EPR line shape and, thus, the rotational correlation time noticeably. We rationalized this finding to the local disordering at the label site due to polymer conjugation, so that line broadening due to increasing molecular weight upon PEGylation was cancelled by the decrease in linewidth due to the enhanced local disordering.[102] Here CW EPR serves as a “scale” or

"balance" to weight the conjugates formed by proteins and polymers as well as a probe to sense the local structural disruptions caused by polymer conjugation (Figure 8).

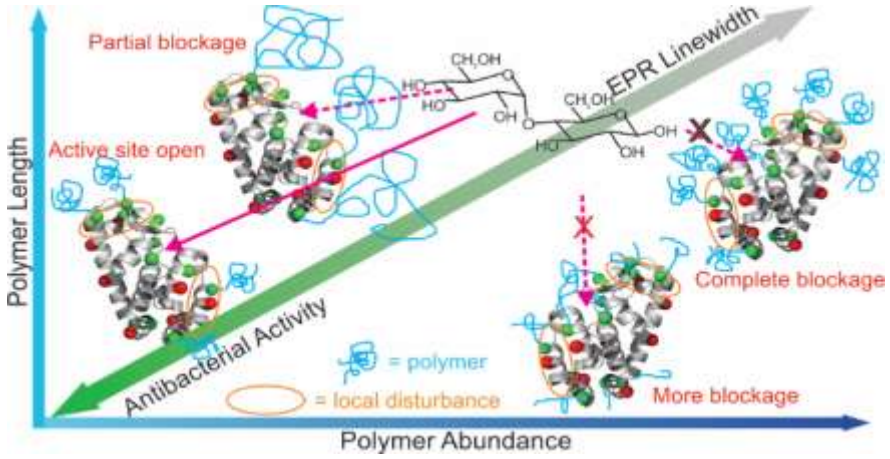

Figure 8. A comprehensive understanding on the conjugation of polymer onto proteins and its impact on CW EPR linewidth and protein catalytic activity. Figure obtained from reference with permission.[102]

3.5 CW EPR to probe polymeric micelle's interior: crowding and polarity

Polymeric micelles, including the recently developed invertible micelle assemblies (IMAs),[103, 104] are advanced molecular carriers. The cargos, although in most cases are small drug molecules, have been expanded to peptides, proteins, and nucleic acids, impacting biomedicine and possibly gene editing.[105] The interior of the micelles and the behavior of the large biomolecular cargos such as peptides and nucleic acids upon loading and release are challenging to probe experimentally, especially the polarity and crowding, but are essential to understanding the micelle functionality at the molecular level. By attaching EPR spin probes/labels to a popular therapeutic HA peptide, we were able to use CW EPR to probe the assembling status upon contact with a "bad" solvent, methanol (MeOH), that disassembles micelles as judged by the

change in the mobility and linewidth of the CW EPR spectra on a non-selectively labeled HA peptide.[106] The extents of disassembly of two micelles with different composition and thus, "tightness", was revealed as a function of MeOH contents in the mixture. Recently, by site-specific spin labeling, we were able to monitor the relative local polarity and crowding changes for the same HA peptide in a model IMA upon addition of MeOH and inversion caused by toluene.[107] The inversion of the IMA by toluene caused aggregation of the HA peptide, while the disassembly by MeOH released the peptide into the solvent. The aggregation was probed similarly as in the previous case,[108] while CW EPR was utilized to determine the distances among three labeled HA peptide residues, one at a time, in toluene, to probe the aggregation.[107] Using the "ShortDistance" program by Dr. Altenbach,[25] we found that the "head" of the peptide tends to aggregate closer to each other as compared to the "end" of the peptide in toluene (Figure 9), based on which an aggregation state model was proposed for the HA peptide in IMA. This work is important for understanding the behavior of large biomolecular cargos in micelles, shedding light on the rational design of advanced micelles for optimal drug delivery performance. CW EPR not only serves as the sensors for peptide cargo loading and release but also offers a direct observation of peptide aggregation. We also proved that CW EPR can directly probe the crowding and local environment of polymeric micelles using spin labeled polymers.[109]

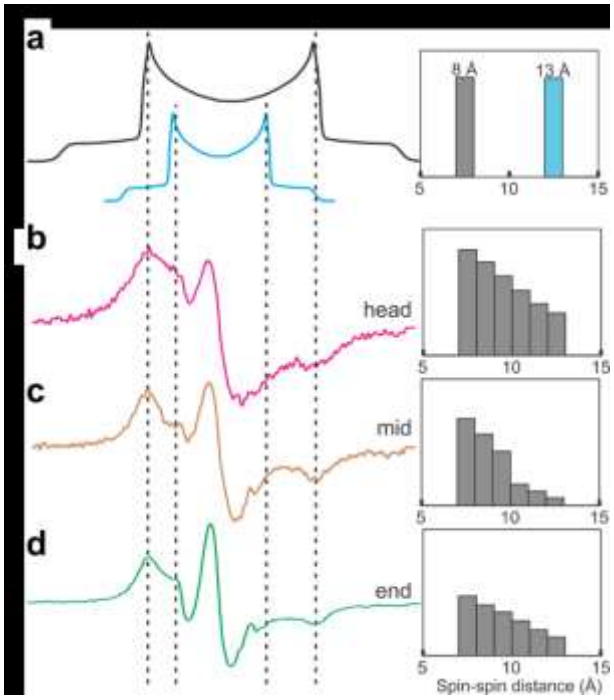

Figure 9. Spin-labeled peptide aggregation states probed with CW EPR based short distance measurements. (a) Theoretical calculation of the Pake patterns for 8 and 13 Å. (b-d) Experimental CW EPR data (scan range 150 G centered at ~3400 G) show different lineshapes which are composed of the regular three-line splitting of nitroxides as well as the Pake pattern due to the spin-spin magnetic dipolar interactions under close spin proximity caused by aggregation. The estimated distance distribution among spin labels at the head (b), mid (c), and end (d) positions of the peptide are shown in the inset of each. Figure obtained from reference with permission.[107]

3.6 CW EPR in food protein studies.

The classic application of CW EPR in food science was to probe the presence and quantity of radicals during food processing and degradation. SDSL of food proteins, such as beta-glutamine and lysozyme, in combination with CW EPR spectroscopy, should in principle facilitate the

understanding of the function of food proteins by providing the structural basis underlying the food processes or upon food protein alteration.[110-112] The principle is similar to that discussed in 3.4, so that CW EPR can probe the size/weight changes of food proteins upon modification as well as the local structural disturbance due to the modification. Upon interaction with other species in food systems, CW EPR can also offer the contact pattern/region as well as the dynamics changes upon contact, as discussed in 3.1.

4. Future perspectives and more dimensions.

Most of the applications our team has explored were based on a single biomolecule and a single synthetic material. We foresee a possibility of including more components in both sides of the interaction counterparts. For example, protein-protein, protein-DNA, and other protein binding partner interaction systems can be loaded into a confinement environment and investigate the influence of spatial restriction on the interaction using all established CW EPR techniques. The results can provide in-depth understanding of how proteins interact with other species near the physiological environment and help the rational design of drug molecules that target these protein interaction systems. The materials matrices can also be more sophisticated. For examples, under spatial confinement, molecular crowders can also be included,[72, 73, 113] which offers a closer mimicking of the cellular environment. CW EPR will provide unique information of the involved biomacromolecules including but not limited to the dynamics and polarity of a labeled site(residue), the strength of the interaction at the labeled site(residue), as well as the crowdness/aggregation and even the molecular weight of the labeled molecule (in certain circumstances).

The selection in spin labels can also be varied to facilitate the research depending on the goals and systems, possibly offering a fourth dimension to our Figure 1. For example, spin labels that do not require disulfide linkage and/or are stable under the reducing conditions will allow for

investigation closer to the cellular conditions or directly in cells.[37, 114-120] Other nitroxides with different rigidity or relaxation properties can also be employed for structure and dynamics studies.[121-126] For cysteine-rich proteins, unnatural amino acids can be employed.[127-129] The linker's flexibility may limit local dynamics measurement yet valuable information such as the local polarity and crowdness can still be revealed. Cu^{2+} spin probe is another option when combined with the NTA linker to enable site-specific Cu^{2+} labeling.[130-132] Recently it has been demonstrated that CW EPR offers the local dynamics of the Cu^{2+} site.[131]

Of course, pulsed EPR technique can also be included so that more structural information on the target system, such as large-scale intramolecular distance changes upon interaction with synthetic materials (probed by double electron-electron resonance, DEER, or double quantum coherence, DQC, relaxation enhancement, and RIDME),[125, 133-147] the water accessibility and local metal coordination (probed by ESEEM/HYSCORE/DNP),[148-154] and conformational dynamics (probed by Saturation Recovery, SR)[155-157] can be determined. All efforts will result in a more thorough understanding on the target systems.

5. Summary

In this perspective, based on our experience and preliminary findings, we highlight the possible directions of expanding the applicability of CW EPR spectroscopy, focusing on the interface of synthetic materials at the nanoscale and biomolecules, especially protein and peptide, with EPR spin labeling. The goal is to maximize the use of CW EPR and nitroxide spin labeling to advance more research areas such as in-cell mimicking, hybrid biocatalysts, molecular carrier, and nano-bio interface. All discussions in Section 3 were probed preliminarily in the past 5-6 years since the beginning of the establishment of our team; there is no doubt that more in-depth studies can be carried out to yield more in-depth understanding in the 6 areas (3.1-3.6). By varying the

selection of spin labels and including various pulsed EPR techniques at different frequencies, which are not difficult to access nowadays,[158] EPR at the interface of the two kingdoms, synthetic nanostructures and biomacromolecules, will yield even more fruitful scientific outcomes. Lastly, although a PI's research directions should be driven by his/her interests in science (and should not be limited by equipment/instrument), practical barriers need to be overcome first. Thus, we hope to offer some encouragement and excitement to EPR scientists at the PhD and postdoctoral level that even without requesting multi-million dollar startup funds, it is still possible and promising to carry out EPR research and impact a broad spectrum of science and thus, initiate an independent academic career.

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